

## METHODOLOGICAL ARTICLE

# Thrombin generation by calibrated automated thrombography in goat plasma: Optimization of an assay

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### Abstract

The goat model of atrial fibrillation (AF) allows investigation of the effect of AF on coagulation. However, assays for goat plasma are not available from commercial sources. Calibrated automated thrombography (CAT) provides a global view of the coagulation profile by assessing *in vitro* thrombin generation (TG). We describe the customization of the CAT assay in goat platelet-poor plasma (PPP) and in factor Xa (FXa)-inhibitor-anticoagulated PPP. TG was initiated in the presence of phospholipids and either (a) PPP reagent, reagent low, or reagent high; (b) goat brain protein extraction (GBP); or (c) Russell's viper venom-factor X activator (RVV-X). Contact activation was assessed by adding corn trypsin inhibitor. Different concentrations of prothrombin complex concentrate (PCC) were used to determine the sensitivity of both the GBP and RVV-X method. To obtain FXa-inhibitor anticoagulated plasma, rivaroxaban was added to plasma. TG settings with human reagents were not suitable for goat plasma. TG triggered with GBP increased peak height and ETP values. Similarly, the RVV-X method produced comparable TG curves and was more sensitive to PCC titration. Finally, both methods were able to detect the decrease in clotting potential induced by FXa inhibition. This is the first study that reports the customization of the CAT assay for goats. The GBP and RVV-X methods were comparable in triggering TG in goat plasma. The RVV-X method seemed to better discriminate changes in TG curves due to increases in clotting potential as well as to FXa inhibition by rivaroxaban in goat plasma.

### KEYWORDS

goat, plasma, rivaroxaban, thrombin generation, thrombography

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## Essentials

- Optimized clotting assays for the goat model are needed to study the effect of atrial fibrillation on coagulation.
- Blood clotting assays for goats are not available.
- Settings of the blood clotting assay were optimized for goat and compared to human conditions.
- Two different methods were developed to measure blood clotting in goats.

## 1 | INTRODUCTION

Atrial fibrillation (AF) is one of the most common forms of sustained arrhythmias in clinical practice.<sup>1</sup> AF is associated with an increased risk of stroke and other thromboembolic events as a consequence of abnormal changes in blood flow, the vessel wall, and, most importantly, the hemostatic system.<sup>2</sup> People with chronic AF exhibit a hypercoagulable state that is characterized by increased plasma levels of pro-thrombotic markers (e.g., prothrombin fragment 1.2, thrombin-antithrombin complexes and fibrin turnover).<sup>3</sup> Moreover, young paroxysmal AF patients with low risk for stroke display increased factor (F) IXa-AT plasma levels as a reflection of a pre-thrombotic state induced by “lone AF” (i.e., AF without underlying structural heart disease).<sup>4</sup>

However, little is known about the molecular mechanism by which the coagulation system becomes activated during AF, nor on the effects of activated coagulation factors on cardiac remodeling and AF progression.<sup>5</sup>

To unravel these critical aspects, our group uses an experimental goat model in which AF is induced by atrial burst pacing, leading to a progressive increase in AF episode duration until AF does not terminate spontaneously anymore (persistent AF).<sup>6</sup> This model allows the investigation of changes in the heart (e.g., electrical, structural) and blood that occur within days to months of AF.<sup>7,8</sup> Ongoing research by our group focuses on the mechanisms behind the inhibitory effects of anticoagulation on atrial structural remodeling, as previously shown for nadroparin in the AF goat model.<sup>5</sup>

To monitor AF-induced alterations of the coagulation system and to investigate the pathways involved in the AF-related hypercoagulable state, suitable coagulation assays for goat plasma are needed, but these are not available from commercial sources.

Calibrated automated thrombography (CAT) is a well-known functional assay to assess plasma thrombin generation upon *in vitro* activation of the coagulation system.<sup>9,10</sup> It relies on the ability of thrombin to cleave a low-affinity fluorogenic substrate (Z-Gly-Gly-Arg-AMC), thereby allowing continuous recording of thrombin's enzymatic activity.<sup>10</sup> Moreover, the thrombin generation curve reflects the contribution of either the procoagulant or the anticoagulant pathways, providing a global view on the overall coagulation profile of the sample.<sup>11,12</sup> For these characteristics, the CAT assay was selected as primary hemostatic tool to monitor anticoagulant therapy and to assess hypercoagulability in the goat model of AF (unpublished data).

However, the most common reagents used to trigger thrombin generation in the CAT assay are human-derived and/or optimized for human plasma. Therefore, these may not be able to trigger reliable

thrombin generation curves in goat plasma. Moreover, commercially available goat-derived reagents for the CAT assay are lacking. To overcome species-specific differences and reliably measure thrombin generation in goat plasma, modifications of the assay are needed.

This paper describes the customization and optimization of the CAT assay for the assessment of thrombin generation in goat platelet-poor plasma (PPP). Furthermore, it reports the optimal test conditions for the evaluation of the clotting potential in FXa-inhibitor-anticoagulated goat plasma.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Blood samples and brain tissues were collected from untreated female Dutch milk goats with no atrial fibrillation. Average body weight was  $68.5 \pm 11.6$  kg and average age was  $2.8 \pm 1.1$  years.

Animal procedures were conducted in accordance with national and institutional guidelines for the use of laboratory animals and were approved by the local ethics committees for animal experiments of Maastricht University (DEC2014-025).

### 2.2 | Blood collection and plasma preparation

Blood was collected from the jugular vein using BD Precision Glide needles and BD Vacutainers (3.2% [w/v] citrate). The first 10 ml of venous blood was discarded. Goat PPP was obtained following two centrifugation steps at room temperature: the first at 2000g for 5 minutes (min) and the second at 11,000g for 10 min, according to our in-house protocol.<sup>13</sup> Goat normal pooled plasma (GNP) was obtained by pooling together the PPP obtained from 12 goats. Plasma aliquots were stored at  $-80^{\circ}\text{C}$  until use.

Human normal pooled plasma (HNP) was prepared in-house by pooling plasma from at least 80 healthy volunteers not using any medication, as described previously.<sup>13</sup>

### 2.3 | Goat brain protein extraction

Goat brain specimens were freeze-dried. Freeze-dried tissues were brought to room temperature and grinded. The powder was dissolved in a solution of N-Octyl-B-D-Glucopyranoside (50 nM, Sigma-Aldrich) and vortexed for 30 min. The preparation was then

centrifuged at maximum speed (13,000g) for 10 min at room temperature. The supernatant was transferred into a new microcentrifuge tube and centrifuged a second time as described previously.

Different dilutions of goat brain protein extraction (GBP) were obtained by diluting the GBP stock solution with Hepes NaCl buffer (Sigma-Aldrich). A protein assay (Bio-Rad) was used to quantify the total protein content in 50-times-diluted GBP preparation, which was found to be equal to 16.6 mg/ml.

## 2.4 | Thrombin generation

Thrombin generation was measured by means of the CAT method.<sup>13</sup> Fluorescence was measured in an Ascent Reader (Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460-nm filter set, and thrombin generation curves were calculated using Thrombinoscope software (Thrombinoscope B.V., Maastricht, The Netherlands). Correction for inner filter effects and substrate consumption was performed by calibrating the results from each thrombin generation analysis against the fluorescence curve obtained from the same plasma with a fixed amount of calibrator (Thrombin Calibrator, Thrombinoscope B.V.).

Unless stated otherwise, measurements were conducted in triplicate on 80  $\mu$ l of goat or human PPP in a total volume of 120  $\mu$ l (20  $\mu$ l fluorogenic substrate, calcium chloride, and 20  $\mu$ l trigger reagent).

Thrombin generation was initiated by adding either (a) PPP reagent, reagent low, or reagent high, containing human recombinant TF (Stago); (b) GBP; or (c) Russell's viper venom-factor X activator (RVV-X, ITK Diagnostics BV) in the presence of phospholipids (PL, Avanti Polar Lipids). Measurements performed in human plasma with PPP reagent low, PPP reagent, or PPP reagent high, were comparable to reactions performed with in-house reagents using 1, 5, or 20 pM as final TF concentration, respectively.

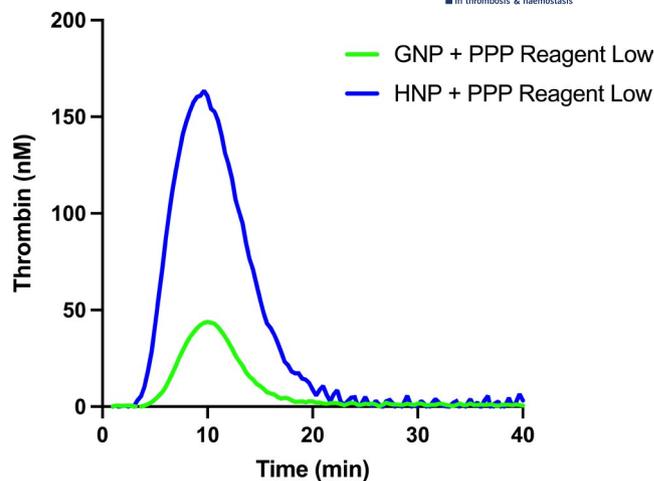
Inhibition of the intrinsic pathway was achieved by adding corn trypsin inhibitor (CTI, Haematologic Technologies) to the reaction mixture. Different concentrations of prothrombin complex concentrate (PCC, Sanquin Plasma Products B.V.), were used to increase the TG in goat plasma. To obtain FXa inhibition, anticoagulated plasma rivaroxaban (Bayer AG) was added to goat plasma before the assay.

## 3 | RESULTS

### 3.1 | PPP reagent low-induced thrombin generation in goat plasma

TF is one of the most common triggers used to initiate thrombin generation in the CAT assay. Depending on the concentration of TF, the sensitivity of the assay can be adjusted to a specific coagulation (and/or anticoagulant) pathway.

Because the CAT triggered by low levels of TF has been shown to activate both intrinsic and extrinsic pathways, this TF concentration was chosen to study the thrombin generation in goat plasma.



**FIGURE 1** Calibrated automated thrombography (CAT) in human and goat normal pooled plasma. Thrombin generation was triggered by adding PPP reagent low to human (HNP, blue curve) and goat (GNP, green curve) plasma (average curves of  $n = 3$ )

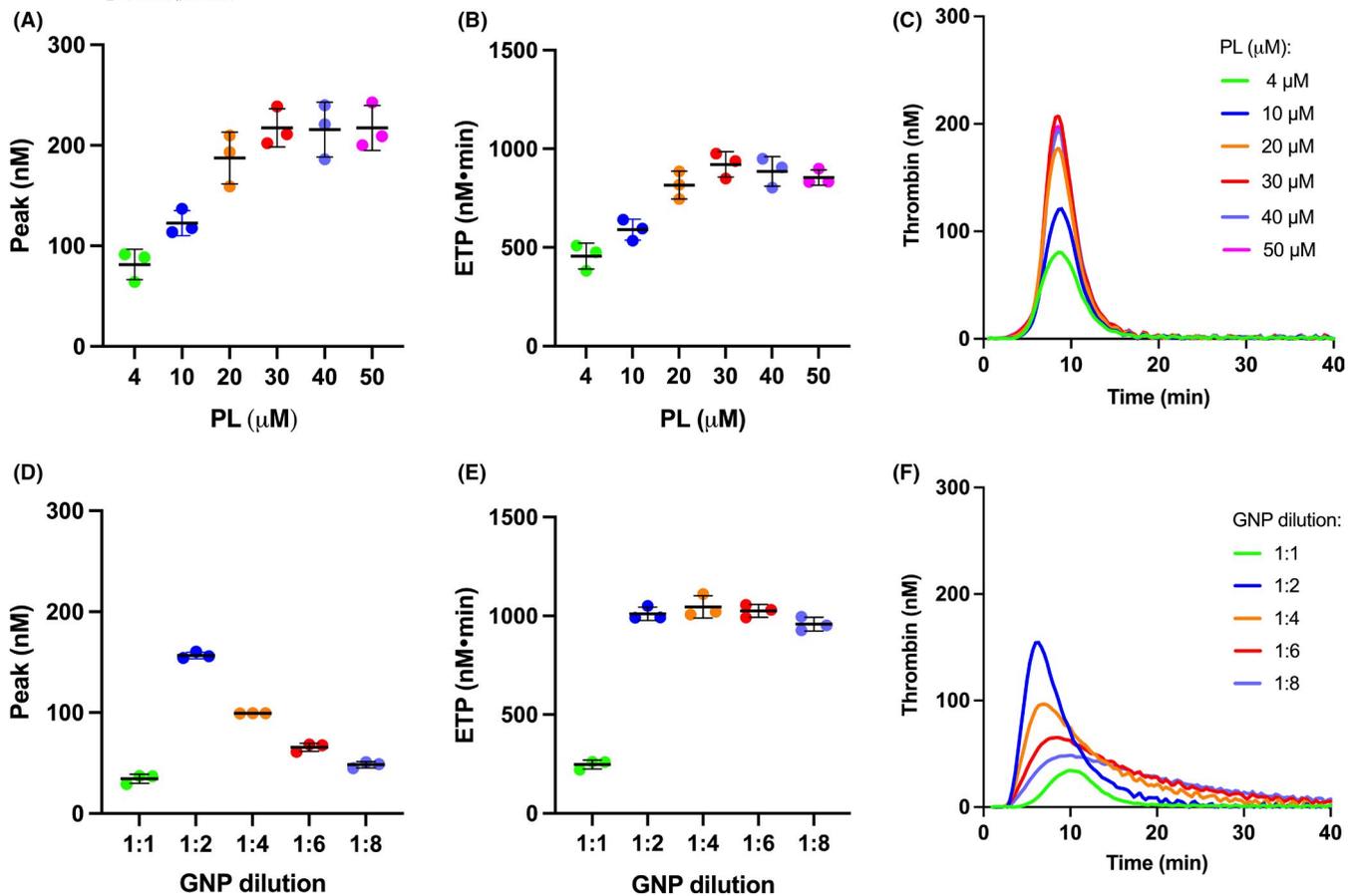
Initially, because of a lack of goat-derived reagents, thrombin generation was initiated with in-house reagents containing human TF. However, the thrombin generation curve obtained in goat plasma showed substantially lower ETP ( $308.2 \pm 23.8$  nM/min) and peak height ( $44 \pm 4.9$  nM) values than in human plasma (ETP:  $1361.7 \pm 45.7$  nM/min, peak:  $159.2 \pm 5.9$  nM) and as such, was unsuitable for further reliable measurements (Figure 1).

### 3.2 | Effect of increasing phospholipids concentration and plasma dilution on thrombin generation

To raise the amount of generated thrombin in goat plasma, we increased the concentration of PL in the reaction mixture. As shown in Figure 2A,B, PL concentrations greater than 4  $\mu$ M produced an increase of both peak height and ETP of the TG curves. The highest increase in both parameters (peak:  $217.3 \pm 18.9$  nM and ETP:  $921.1 \pm 64.7$  nM/min) was reached at 30  $\mu$ M of PL, whereas lag time was not affected.

At low TF concentrations, high PL concentrations tend to enhance the contribution of contact activation. Under these circumstances, thrombin generation is highly dependent on the activation of the intrinsic coagulation pathway (Figure 2C).<sup>14</sup>

To optimize the thrombin generation curve and develop a CAT equally sensitive to pro- and anti-coagulant forces, measurements were performed in diluted plasma. As reported by Tchaikovski et al., reactions carried out in diluted mouse plasma helped to overcome the activity of natural coagulation inhibitors (e.g., TF pathway inhibitor and antithrombin) and to increase the peak height and ETP of the thrombin generation curves.<sup>15</sup> Figure 2D shows that, in goat plasma, the largest increase in peak height was achieved at a plasma dilution of 1:2 ( $156.6 \pm 3.2$  nM, 4.5-fold compared with nondiluted plasma). At this dilution, the lag time shortened by 1.6-fold. At higher plasma



**FIGURE 2** Effect of PL titration and plasma dilution on thrombin generation in goat plasma. Thrombin generation was measured in the presence of PPP reagent low and 4, 10, 20, 30, 40, and 50  $\mu\text{M}$  of PL (A–C). Effect of PL titration on peak height (A), ETP (B), and TG average curves (C). Thrombin generation was measured in diluted GNP plasma (1:1, 1:2, 1:4, 1:6, 1:8) in the presence of PPP reagent low (D–F). Effect of plasma dilutions on peak height (D), ETP (E), and TG average curves (F). Results are expressed as mean  $\pm$  SD,  $n = 3$

dilutions, peak heights dose-dependently decreased while ETP values remained stable (Figure 2D,E).

As reported by Tchaikovski et al., plasma dilutions are expected to reduce the contribution of the natural coagulation inhibitors in mouse plasma.<sup>15</sup> In goat plasma, this effect was not tested. However, to exclude this possibility and to increase the TG parameters without affecting the sensitivity of the assay to the anticoagulant pathways, we decided to explore alternative strategies.

### 3.3 | Goat brain protein extraction-induced thrombin generation

As one of the most highly vascularized organs of the body, the brain contains large amounts of TF, which can be extracted and used to initiate coagulation *in vitro*.

Figure 3A–C shows thrombin generation initiated in goat plasma with 4  $\mu\text{M}$  of PL and different dilutions of GBP used as a source of TF. To obtain an estimate of the concentration of goat TF, the TG curves were compared with the curves obtained in human plasma in the presence of 4  $\mu\text{M}$  of PL and increasing concentrations of human TF.

The results show that thrombin formation initiated with 400-, 200-, and 50-times-diluted GBP was reproducible (Table S1) and comparable to thrombin generated in human plasma triggered with PPP reagent low, PPP reagent, or PPP reagent high, respectively.

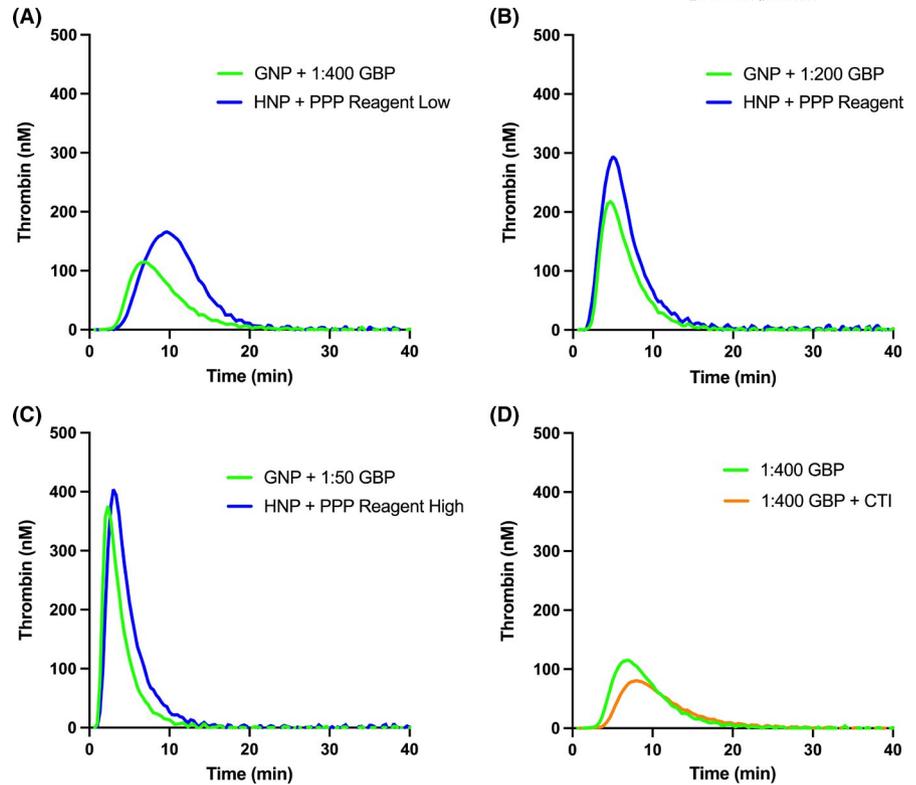
To rule out contact activation, the goat intrinsic pathway was inhibited by CTI. Figure 3D indicates a slight decrease in thrombin generation when CTI was added to the reaction mix, suggesting that GBP may lead to activation of the goat contact system.

### 3.4 | Improving assay sensitivity: GBP vs RVV-X-induced thrombin generation

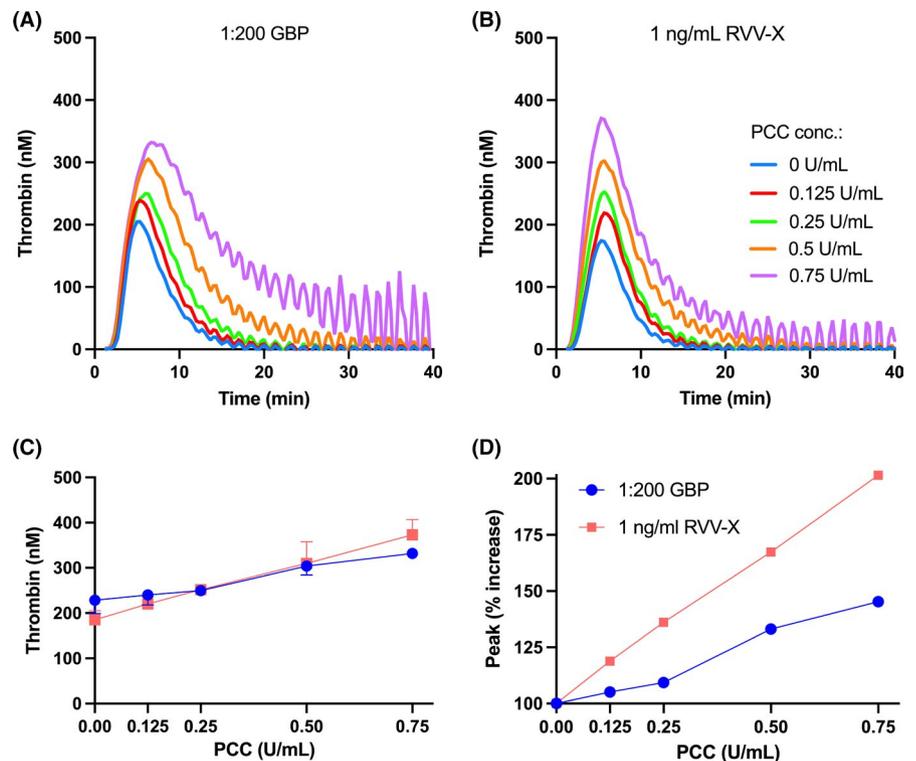
Once the optimal conditions for a reliable thrombin generation curve were established, the assay sensitivity to small increases in the goat clotting potential was tested.

To enhance the thrombin formation in GNP, increasing concentrations of PCC were added to the reaction mixture. Thrombin generation was initiated with diluted GBP. Subsequently, the RVV-X activator, a known exogenous activator of coagulation that cleaves and activates FX into FXa, was used as alternative trigger (Table S2).<sup>16</sup>

**FIGURE 3** Thrombin generation induced by goat brain protein extraction (GBP). Thrombin generation was initiated with in-house reagents PPP reagent low, PPP reagent, or PPP reagent high in HNP plasma (blue curve) and with 400-, 200- and 50-times-diluted GBP in GNP plasma (green curve) (A-C). Effect of CTI on thrombin generation induced by GBP (D). All curves are average curves of  $n = 3$

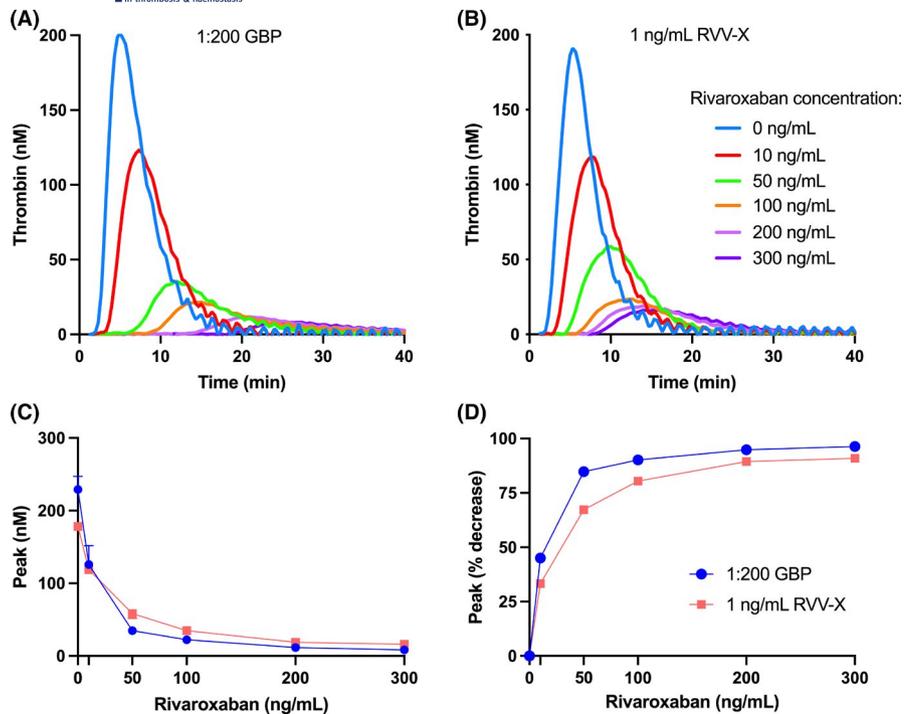


**FIGURE 4** Effect of PCC titration on thrombin generation. Thrombin generation was initiated with 200-times-diluted GBP and 4  $\mu$ M of PL or 1 ng/ml of RVV-X and 4  $\mu$ M of PL in GNP plasma. All curves are average curves. The legend in panel B also applies to panel A. Effect of PCC titration on Peak height values obtained with GBP (blue) and RVV-X (orange) (C). Percentage of increase of peak height values relative to 0 U/ml of PCC (D). All curves are average curves. The legend in panel D also applies to panel C. Results are expressed as mean  $\pm$  SD



As illustrated in Figure 4A,B, both methods were able to detect the increments of generated thrombin induced by PCC titration. Comparable peak height values were reached with both methods at different PCC concentrations (Figure 4C). However, the RVV-X method resulted in a higher relative peak increase compared with

the GBP method (peak % increase at 0.75 U/ml of PCC, 201% and 145%, respectively; Figure 4D). This indicates that the RVV-X method may better discriminate between TG curves that reflects small changes of the goat clotting potential compared with the GBP method.



**FIGURE 5** Effect of rivaroxaban titration on thrombin generation. Thrombin generation was initiated with 200-times-diluted GBP and 4  $\mu$ M of PL or 1 ng/ml of RVV-X and 4  $\mu$ M of PL in GNP plasma. All curves are average curves. The legend in panel B also applies to panel A. Effect of rivaroxaban titration on peak height values obtained with GBP (blue) and RVV-X (orange) (C). Percentage of decrease of peak height values relative to 0 ng/ml of rivaroxaban (D). All curves are average curves. The legend in panel D also applies to panel C. Results are expressed as mean  $\pm$  SD

### 3.5 | Thrombin generation in anticoagulated goat plasma

In a previous study by our group (unpublished data), goats with AF received oral rivaroxaban treatment (3 mg/kg twice daily). To establish the optimal assay conditions to measure the thrombin generation in anticoagulated goat plasma, rivaroxaban was added to the GNP before the measurement. To assess which of the two methods made the assay more sensitive to rivaroxaban titration, thrombin generation was measured in the presence of either GBP or RVV-X (Figure 5A,B). Both methods appeared to detect the decreases in peak height values caused by rivaroxaban (Figure 5C). However, the RVV-X method seemed to better discriminate the TG curves at rivaroxaban concentrations ranging between 10 and 200 ng/ml, as indicated by the smaller peak percentage decrease compared to the one obtained by the GBP method (peak percentage decrease at 50 ng/ml rivaroxaban, 67% and 85%, respectively; Figure 5D).

## 4 | DISCUSSION

In this study, we reported different methods for the customization and optimization of the CAT assay in goat PPP. Furthermore, we identified the optimal assay conditions to evaluate thrombin generation in anticoagulated goat plasma.

The goat model is a well-established and extensively researched model for the pathogenesis of AF.<sup>6</sup> Because AF can be maintained in the goat for several months, this animal model has also become an interesting tool to investigate the long-term effects of AF on the coagulation system. Moreover, it has been used to elucidate

the effect of FXa inhibition on atrial structural remodeling and AF progression.<sup>5</sup> For these reasons, it has become crucial to develop hemostatic assays suitable for goat plasma.

The CAT assay is able to reliably assess changes of the clotting potential and monitor anticoagulant therapy.<sup>17</sup> Additionally, unlike other techniques (e.g., ELISAs), it does not require many species-specific reagents, which makes this test easier to be performed in goat plasma. The biggest advantage of the CAT is to provide a complete view on the coagulation profile of the sample by evaluating the contribution of both pro- and anti-coagulant pathways.<sup>10</sup>

Depending on the type and concentration of the trigger, the sensitivity of the CAT can be adjusted to a specific (pro- or anti-coagulant) pathway. In this study, we aimed to establish the optimal assay conditions to equally evaluate the contribution of extrinsic and intrinsic pathways of the goat coagulation system.

Very little is known about the goat hemostatic system or about differences and/or homologies between the goat and the human coagulation factors. Studies performed in the 1970s reported that overall coagulation values were similar between the two species. However, the goat plasma had a shorter activated thromboplastin time and a longer thrombin time compared with human plasma according to the measurements of the time.<sup>18,19</sup> In our study, we observed that the standard settings developed to measure thrombin generation in human plasma did not yield reliable thrombin generation curves in goat plasma. This may be explained by the fact that the human TF in PPP reagents may not have a high affinity for the goat FVIIa. In fact, goat and human TF share 68.4% of identity, whereas goat and human FVII only 66.1%.<sup>20</sup>

To increase the amount of generated thrombin, we explored different strategies. In line with previous studies on human and murine plasma, performing the assay with increased concentrations

of PL or with diluted plasma seemed to enhance goat thrombin generation.<sup>15,21</sup> However, increasing PL concentrations may make the assay highly dependent on the intrinsic pathway, whereas plasma dilution may decrease the assay sensitivity to anticoagulation pathways.<sup>14</sup>

To keep the CAT assay equally sensitive to all coagulation processes, we initiated thrombin generation with GBP as source of TF.

Interestingly, GBP led to a reliable increase in peak height and ETP values. Our data revealed that this assay modification provided a reproducible method to perform the CAT assay in goat plasma. However, it also showed a few limitations. First, this method can only be carried out if the source of goat TF is available (e.g., goat brain or other TF-rich goat organs). Second, we were not able to purify nor to assess the exact concentration of goat TF in the whole protein extraction. This means that GBP preparations may contain other membrane proteins (e.g., thrombomodulin) and lipids that may affect the assay sensitivity. Such observation is also supported by the fact that GBP seems to also trigger goat contact activation.

To overcome these limitations, we explored another assay modification. Thrombin generation was initiated with RVV-X. This metalloprotease cleaves FX into FXa and activates the common coagulation pathway.<sup>16</sup> Despite the fact that this is a nonstandardized method and does not reproduce a common mechanism of activation of the coagulation system, the RVV-X method yielded good thrombin generation curves. Furthermore, this method seemed to better discriminate small increases of clotting potential as compared to the GBP method.

Finally, we established the most sensitive settings to measure thrombin generation in FXa-inhibitor-anticoagulated goat plasma. To do so, we compared the curves obtained with the GBP and the RVV-X method in the presence of rivaroxaban. Our findings showed that both methods were able to detect the decrease in clotting potential produced by FXa inhibition. However, the RVV-X method better discriminated the decrease in peak height induced by rivaroxaban.

## 5 | CONCLUSIONS

To the best of our knowledge, this is the first study that reports the customization and optimization of the CAT assay for a large animal model. Two distinct methods, GBP and RVV-X, were established and found to be comparably able to trigger thrombin generation in goat plasma. Ultimately, the RVV-X method seemed to better discriminate changes in TG curves induced by small increases in clotting potential as well as by FXa inhibition by rivaroxaban in goat plasma.

### RELATIONSHIP DISCLOSURE

H.t.C. received consultancy fees from Bayer, Pfizer, Leo, and Portola/Alexion and received research grants from Bayer, unrelated to the present work. H.t.C. is a consultant for Alveron and shareholder of CoagulationProfile, a spin-off company of Maastricht University. U.S. received consultancy fees or honoraria from Università della

Svizzera Italiana (USI, Switzerland), Roche Diagnostics (Switzerland), EP Solutions Inc. (Switzerland), Johnson & Johnson Medical Limited (United Kingdom), and Bayer Healthcare (Germany). U.S. received research grants from Roche and EP Solutions. Bayer Healthcare (Germany) supported pharmacokinetic study of this investigation. U.S. is cofounder and shareholder of YourRhythmics BV, a spin-off company of University Maastricht.

### AUTHOR CONTRIBUTIONS

Elisa D'Alessandro and Billy Scaf were responsible for the execution of the study and all the experimental work. Rene van Oerle and Arne van Hunnik provided guidance on technical aspects and animal handling. Frans A. van Nieuwenhoven, Sander Verheule, Ulrich Schotten, Hugo ten Cate, and Henri M.H. Spronk were involved in the supervision and coordination of the study. All authors participated in preparing and revising the manuscript.

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#### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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